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Award Number: W81XWH-12-1-0071

TITLE: Microtubule Control of Metabolism in Prostate Cancer

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Bethlehem, PA 18015

REPORT DATE: June 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
June 2013	Annual	1 June 2012 – 31 May 2013

4. TITLE AND SUBTITLE

Microtubule Control of Metabolism in Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-12-1-0071

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Lehigh University
Bethlehem, PA 18015

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The current standard chemotherapy treatment for metastatic castrate-resistant prostate cancer is the microtubule-stabilizing drug, docetaxel, but the median increase in survival is limited to several months. One avenue for improved treatment outcome is to find agents that increase cell death when combined with docetaxel.Here we tested whether two metabolic inhibitors, metformin or 2-deoxy-glucose, function synergistically with docetaxel to block prostate cancer cell proliferation. LNCaP and PC-3 prostate cancer cell lines were incubated in drugs targeting the microtubule cytoskeleton (docetaxel, paclitaxel, or nocodazole) singly, or in combination with metabolic inhibitors (metformin or 2-deoxy-glucose). Microtubule-targeted drugs, which either stabilize or destabilize microtubules, acted synergistically with either metformin or 2-deoxy-glucose to block cell proliferation and cause cell death in both prostate cancer cell lines tested. Synergy was consistently calculated at the ED50 for each drug, but for many drug combinations, the combination index shifted toward antagonism at higher drug doses. Microtubule stabilizers (docetaxel, paclitaxel) showed greater synergy than did a microtubule destabilizer (nocodazole) when combined with either of the metabolic inhibitor tested. The more-than-additive cell killing measured in two commonly used prostate cancer lines treated with a mixture of microtubule-targeted drugs and metabolic inhibitors indicates that these drug combinations could provide more effective tumor cell killing than either drug alone.

15. SUBJECT TERMS
None provided.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	18	19b. TELEPHONE NUMBER *(include area code)*

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Introduction

Prostate cancer is the second leading cause of cancer deaths for men in the United States (American Cancer Society, 2013 statistics). Although often cured by surgery or radiation treatment, for many patients the disease progresses from one responsive to androgen removal to one where cancer cells have become androgen insensitive, the latter termed metastatic castrate-resistant prostate cancer (mCRPC). The current chemotherapy treatment for mCRPC is docetaxel, but this drug treatment is not very effective, increasing lifespan by an average of 3 months. Docetaxel (Taxotere™) and other taxanes are drugs that act by stabilizing microtubules of the mitotic spindle, blocking cell division and ultimately leading to cell death. An alternative to blocking cells in mitosis is to inhibit intracellular metabolism. Many cancers depend on glycolysis for most ATP production (the Warburg effect) and these high glycolytic rates also fuel the pentose phosphate pathway for biosynthesis of nucleosides and amino acids, suggesting that metabolic inhibitors may induce cell death in cancer cells, and this has been demonstrated in several prostate cancer cell lines treated with 2-deoxyglucose (2-DG), an inhibitor of glycolysis. Metformin is another metabolic regulator currently under study as an anticancer drug because of the strong epidemiological data showing that diabetics taking metformin have a significantly reduced risk of cancer development, compared to diabetics taking other types of medication. The experiments performed here were designed to test whether the combination of microtubule-targeted drugs and metabolic inhibitors act synergistically (more-than-additive) to inhibit proliferation of human prostate cancer cells grown in cell culture.

Keywords: combination index, microtubule, tubulin, docetaxel, metformin, 2-deoxyglucose

Body

The original two goals summarized in the SOW were to address the following questions:
A. Does increased microtubule stability decrease glycolytic rate or the pentose phosphate pathway?

B. Does microtubule stabilization with docetaxel synergize with metformin or 2-deoxy-glucose to inhibit cell proliferation and/or induce cell death?

We opted to begin with Goal B because completion of this goal would tell us whether testing a possible mechanism responsible for the synergy (Goal A) was necessary. Given the 1 year time frame of the Exploration-Hypothesis award, we were able to thoroughly explore Goal B, but did not have the time or resources to explore Goal A. We also realized that we would need to scale back to 2 cell lines in order to conduct experiments that thoroughly explored each drug concentration series and that allowed sufficient time to replicate all experiments several times for confidence in the reproducibility and statistical significance of the work.

Summary of Results

1. Microtubule stabilizing drugs (docetaxel and paclitaxel) act synergisically with the metabolic inhibitor metformin to inhibit proliferation of LNaP and PC-3 cancer cell lines.

Methods: Cell proliferation was assayed in triplicate for each drug concentration per experiment, and all experiments were repeated at least 3 times. Proliferation was measured by the colormetric MTS assay. Cell death was confirmed by trypan blue exclusion. Interactions between drugs were tested in a constant ratio checkerboard design, and the interaction assigned a Combination Index (CI) value using CalcuSyn software. The effect of the two drugs on cell cycle progression was measured by flow cytometry of DNA content and by immunofluorescence imaging of cells stained with antibodies to tubulin (the subunit of microtubules).

Results: For both LNCaP and PC-3 cells, docetaxel and metformin in combination produced greater cell death than either treatment alone. The two drugs had CI values of 0.67 (PC-3) - 0.86 (LNCaP) at the ED50 (CI values of < 1 indicate drug synergy). Treatment with paclitaxel and metformin also showed synergy (CI values of 0.64

(LNCaP) and 0.72 (PC-3) at the ED50). Both microtubule stabilizing drugs showed the expected cell cycle block and caused cells to form multipolar spindles. These cells were unable to complete mitosis. Metformin did not change cell cycle progression over the range of concentrations tested.

2. Metformin also acted synergistically with a microtubule-destabilizing drug to inhibit prostate cancer cell proliferation.

Methods: Identical to those described above, except cells were treated with nocodazole, a drug that depolymerizes microtubules and also causes cells to block in mitosis of the cell cycle.

Results: Nocodazole treatment yielded the expected mitotic block in both LNCaP and PC-3 cell lines. The CI values for the combination of nocodazole and metformin were 0.79 (LNCaP) and 0.99 (PC-3), demonstrating synergy between nocodazole and metformin in LNCaP cells and an additive interaction in PC-3 cells.

3. Docetaxel synergizes with 2-deoxy-glucose to inhibit prostate cancer cell proliferation.

Methods: Identical to those described under #1, with the exception that 2-deoxy-glucose was used as a metabolic inhibitor.

Results: Both cell lines were sensitive to the combination of docetaxel and 2-deoxy-glucose. CI values were 0.87 (LNCaP) and 0.37 (PC-3), again indicating synergy between the two drugs. 2-deoxy-glucose did not inhibit cell cycle progression at the concentrations tested.

Data supporting the above summary are included in the Appendix of a manuscript submitted for publication.

Discussion: The one year hypothesis-exploration award allowed us to test whether two commonly used chemotherapies are synergistic, additive or antagonistic when used in combination. The tests conducted in cell lines indicate that drugs disrupting microtubule function, and/or blocking cells in mitosis, are more effective when combined with metabolic inhibitors to inhibit prostate cancer cell proliferation.

Key Research Accomplishments

1. Determined dose-response curves for each drug tested (docetaxel, paclitaxel, nocodazole, metformin, 2-deoxy-glucose) in two commonly used prostate cancer cell lines. "Response" was measured as cell proliferation or cell death.
2. Assayed drug combinations in a constant ratio checkerboard design.
3. Determined combination index for all drug combinations tested.
4. Confirmed that microtubule-targeted drugs produced the expected cell cycle block.
5. Confirmed the microtubule-targeted drugs disrupted mitotic spindle morphology.
6. Determined that metabolic inhibitors did not inhibit cell cycle progression.
7. Presented progress in December 2012 at the annual meeting of the American Society for Cell Biology.
8. Submitted manuscript to BMC Cancer.

Reportable Outcomes

Bruce K. Carney and Lynne Cassimeris. 2012. Docetaxel and metformin act synergistically to inhibit growth of prostate cancer cell lines. Mol. Biol. Cell. 23: 869 (abstract #). Included in appendix.

Manuscript submitted to BMC Cancer and included in appendix.

Conclusion

The synergistic increase in cell death measured in prostate cancer cell lines treated with a combination of docetaxel and either metformin or 2-deoxy-glucose suggests that a combination therapy of docetaxel with metabolic inhibitors should be tested in animal models and/or patient trials. A Phase II trial is currently enrolling patients.

References

See references within submitted manuscript (Appendix)

Bruce K. Carney and Lynne Cassimeris. 2012. **Docetaxel and metformin act synergistically to inhibit growth of prostate cancer cell lines.** Mol. Biol. Cell. 23: 869 (abstract #). Presented by BKC at the annual meeting of the American Society for Cell Biology, San Francisco, CA.

Treatments for prostate cancer, one of the most prevalent cancers among men, are an area of ongoing investigation. While docetaxel, a microtubule-stabilizing drug, is effective against many forms of prostate cancer this drug, while the best available treatment to date, shows little improvement in the duration of patient survival (only a few months) in castration resistant prostate cancers. It has been previously shown that increasing microtubule stability (by multiple treatments, including paclitaxel treatment, a natural analog of docetaxel) can lead to a cell cycle delay/mitotic arrest and an increase in cell death. Inhibiting intracellular metabolism has also been proposed as a treatment for multiple cancer types because many cancers favor glycolysis for most of their ATP production (Warburg effect). It has been shown previously that most glycolytic enzymes are localized to the microtubule cytoskeleton; therefore we hypothesize that disturbing the microtubule network should further interfere with ATP production via glycolysis and limit growth of cancer cells. Normal cells, however, should be resistant to these treatments due to their reliance on oxidative phosphorylation. Here we test the hypothesis that there is a synergy between the microtubule stabilizing drug, docetaxel, and drugs that block intracellular metabolism, such as metformin (a drug that inhibits metabolism at multiple points). Cell proliferation was measured for each drug alone and in combination using a colorimetric assay (MTT). While both drugs individually significantly inhibited cell proliferatin, initial results show a super-additive (synergistic) effect between docetaxel and metformin in multiple prostate cancer cell lines including LNCaP (androgen dependent) and PC-3 (androgen independent) cell lines. This synergy both decreases the concentration of each drug needed to inhibit cell proliferation (therefore decreasing possible toxicity) while also increasing the potency of the combination. Based on our results the data suggest a potential new drug combination to great castration resistant prostate cancers.

Submitted to BMC Cancer on July 24, 2013

Title: Microtubule-targeted drugs act in synergy with metabolic inhibitors, metformin or 2-deoxy-glucose, to control growth of LNCaP and PC3 prostate cancer cell lines

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Abstract
Background: The current standard chemotherapy treatment for metastatic castrate-resistant prostate cancer is the microtubule-stabilizing drug, docetaxel, but the median increase in survival is limited to several months. One avenue for improved treatment outcome is to find agents that increase cell death when combined with docetaxel. Here we tested whether two metabolic inhibitors, metformin or 2-deoxy-glucose, function synergistically with docetaxel to block prostate cancer cell proliferation.

Methods: LNCaP and PC-3 prostate cancer cell lines were incubated in drugs targeting the microtubule cytoskeleton (docetaxel, paclitaxel, or nocodazole) singly, or in combination with metabolic inhibitors (metformin or 2-deoxy-glucose). Drugs were assayed in a constant ratio checkerboard design. Cell proliferation/survival was monitored by colorimetric MTS assay, cell cycle distributions by flow cytometry of DNA content, and cell morphology by immunofluorescent images of microtubule and DNA localizations. Cell survival curves over a range of drug concentrations were used to calculate the combination index, a quantitative measure of drug interactions.

Results: Microtubule-targeted drugs, which either stabilize or destabilize microtubules, acted synergistically with either metformin or 2-deoxy-glucose to block cell proliferation and cause cell death in both prostate cancer cell lines tested. Synergy was consistently calculated at the ED50 for each drug, but for many drug combinations, the combination index shifted toward antagonism at higher drug doses. Microtubule stabilizers (docetaxel, paclitaxel) showed greater synergy than did a microtubule destabilizer (nocodazole) when combined with either of the metabolic inhibitor tested.

Conclusion: The more-than-additive cell killing measured in two commonly used prostate cancer lines treated with a mixture of microtubule-targeted drugs and metabolic inhibitors indicates that these drug combinations could provide more effective tumor cell killing than either drug alone.

Keywords (3-10)
combination index, microtubule, tubulin, docetaxel, metformin, 2-deoxyglucose

Background

Prostate cancer is the second leading cause of cancer deaths for men in the United States (American Cancer Society, 2013 statistics). Although often cured by surgery or radiation treatment, for many patients the disease progresses from one responsive to androgen removal to one where cancer cells have become androgen insensitive. This latter stage is referred to as metastatic castrate-resistant prostate cancer (mCRPC). The current chemotherapy treatment for mCRPC is docetaxel, but this drug treatment is not very effective, increasing lifespan by an average of 3 months (reviewed by 1,2). Docetaxel (Taxotere™) and other taxanes are drugs that act by stabilizing microtubules (MTs) of the mitotic spindle, blocking cell division and ultimately leading to cell death (3). In general, other drugs targeting the mitotic phase of the cell cycle have shown only infrequent success in prostate cancer clinical trials, pointing to the need for combination therapies to augment docetaxel (2).

In addition to drugs blocking the cell division cycle, metabolic inhibitors have gained interest recently as potential chemotherapeutics. Many cancers depend on glycolysis for most ATP production (the Warburg effect) and these high glycolytic rates also fuel the pentose phosphate pathway (PPP) for biosynthesis of nucleosides and amino acids (4). These observations have suggested that metabolic inhibitors may induce cell death in cancer cells (5), and this has been demonstrated in several prostate cancer cell lines treated with 2-deoxyglucose (2-DG), an inhibitor of glycolysis (6). Metformin is another metabolic regulator currently under study as an anticancer drug because of the strong epidemiological data showing that diabetics taking metformin have a significantly reduced risk of cancer development, compared to diabetics taking other types of medication (7). Metformin functions at several levels to inhibit metabolism, including inhibiting oxidative phosphorylation in mitochondria and activating AMP-activated protein kinase (AMPK), a cellular energy sensor (7). Use of metformin to treat cancers is currently under investigation, and has shown the ability to induce cell death in cell lines from prostate (6), breast (8) and pancreatic (9) cancers.

Several groups have hypothesized that the MT cytoskeleton contributes to intracellular metabolic regulation (10,11), raising the possibility that docetaxel treatment will be more effective when combined with metabolic inhibitors. In this study we examined whether MT-targeting drugs (including MT stabilizing drugs docetaxel and paclitaxel, and a MT depolymerizing drug, nocodazole) act synergistically with metabolic inhibitors, metformin or 2-DG, to inhibit proliferation and cause cell death in cell lines derived from prostate cancers. Experiments were performed in two commonly used human prostate cancer cell lines, LNCaP and PC-3. LNCaP cells are androgen-sensitive human prostate adenocarcinoma epithelial cells that have a low metastatic potential. PC-3 cells are androgen-insensitive human cells and have a high metastatic potential; these cells are a model for mCRPC (12).

Methods

Cell Culture: Cells were grown at 37□C in a humidified atmosphere of 5% CO_2. LNCaP cells were grown in RPMI-1640 (Sigma-Aldrich) supplemented with 1X antibiotic/antimycotic (Sigma), and 10% fetal bovine serum (FBS) (GIBCO-Invitrogen). PC-3 cells were grown in Nutrient Mixture F-12 Ham (F12K) (Sigma) supplemented with 1.1 g/L sodium bicarbonate, 1X antibiotic/antimycotic (Sigma), and 10% FBS (GIBCO-Invitrogen).

Drug incubations: Cells were plated 24 - 48 hrs before drug addition and were then maintained in the drug for 24 - 72hrs. Docetaxel and metformin were from Tocris, paclitaxel from Molecular Probes, and nocodazole and 2-DG were from Sigma. Docetaxel, pactlitaxel and nocodazole stock solutions were prepared in DMSO; metformin and 2-DG stocks were prepared in dH_2O. For all experiments 0.1% DMSO was used as a vehicle control.

Cell survival assays: Cells were plated in 96-well plates at a density of 5×10^3 cells per well for either cell line. After 24 - 48 hrs, cells were treated with serial dilutions of individual drugs, or drug combinations, as noted. Control wells received an equivalent volume of assay medium containing 0.1% DMSO. All conditions were analyzed in triplicate within a single experiment, and all experiments were repeated 2 or more times, as noted. After 48 - 72 hr incubation, cell viability was quantified using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (MTS; Promega) according to manufacturer's instructions. Briefly, 20mL MTS

solution was added to 100mL medium per well, and plates incubated at 37°C for 1-2 hrs. Absorbance at 490 nm was measured using a Techan Infinite M200pro plate reader driven by I-Control 1.7 software at 10 reads per well. Survival was calculated as a percent compared to control treated cells. To pool data from separate experiments, the absorbance value for DMSO treated cells was set to 2.0 and all values for each drug concentration scaled accordingly. This normalization procedure changed the absorbance value by relatively little since the DMSO treated cells typically had absorbance readings close to 2.0 in each individual experiment.

As a more direct measure of cell death, trypan blue exclusion assays were performed as described previously (13).

Drug synergies: The combination index (CI) was calculated for treatment pairs using CalcuSyn software (Biosoft, Ferguson,MO). The CI provides a quantitative measure of the degree of interaction between multiple agents (14). A CI of <1 denotes synergy, a CI of 1 denotes an additive effect, and a CI of >1 denotes antagonism. Experiments were performed as described above using 4,2,1,0.5, and 0.25 times the calculated median effective dose (ED50) of each drug in a constant ratio checkerboard design. CI values were determined for each individual experiment and are given in the tables as the average of at least 3 experiments per treatment combination.

Indirect immunofluorescence and confocal microscopy: Cells were fixed and imaged as described previously (15). MTs were labeled with a mouse monoclonal α-tubulin (B512; Sigma-Aldrich) and a goat anti-mouse Alexa Fluor 488 (Invitrogen). Propidium iodide (0.15 μM) was used to label DNA. Cells were imaged by confocal microscopy as described previously (15) and images acquired using a 40X/1.3NA objective. Image stacks were converted to maximum intensity projections, exported as TIFF files and assembled using Photoshop.

Results and Discussion:

MT stabilizing drugs, docetaxel and paclitaxel, act synergistically with the metabolic inhibitor, metformin, to inhibit proliferation of LNCaP and PC-3 prostate cancer cell lines.

To address whether prostate cancer cell lines are more sensitive to MT-targeting drugs when combined with metabolic inhibition, we first assayed docetaxel, the primary treatment option for advanced, metastatic prostate cancer (1) in combination with metformin, a metabolic inhibitor (5,7). As shown in Figure 1 (A,B) for cells treated for 48 hr with either drug alone or in combination, LNCaP cells were more sensitive to the combination of docetaxel and metformin than to either drug alone. PC-3 were slightly more sensitive to the drug combination at low concentrations, but at most of the concentrations tested, these cells were inhibited primarily by metformin. A longer incubation of 72 hr showed the same general trends (not shown). For each cell line, cells were more sensitive to increasing metformin concentrations than to increasing docetaxel concentrations for the ranges tested (Figure 1 A,B). Additional experiments testing higher concentrations of docetaxel (up to 1.6 μM) did not cause significantly greater cell death than that shown in Figure 1.

The commonly used MTS assay for cell viability measures the activity of intracellular dehydrogenases as a proxy for cell health and cell number. To confirm that the above results reflect cell death, we used trypan blue uptake as a more direct measure of cell death. As shown in Figure 1 C,D, docetaxel (50 nM; approximately one-half the ED50 for cell death) or metaformin (1.5 mM; approximately one-half the ED50 for cell death) increased the percent of trypan blue positive LNCaP and PC-3 cells over that seen in DMSO treated controls. The combination of these drugs (50 nM docetaxel and 1.5 mM metformin) increased the percentage of dead cells in each cell line. For LNCaP cells, treatment with the drug combination was nearly twice the sum of the individual drug treatments. For PC-3 cells, the drug combination resulted in a slightly greater percentage of dead cells compared to the sum of the individual drug treatments.

To test quantitatively whether docetaxel and metformin act synergistically in LNCaP or PC-3 cells, CI values were calculated for cells treated with these drugs. As shown in Tables 1 and 2, the combination of docetaxel and metaformin increased cell death synergistically, with a greater synergy observed in LNCaP cells compared to PC-3 cells at the ED50. At the EC75 and EC90, the drug combination shifted toward additive and antagonistic effects, similar to previous results reported for PC-3 cells treated with a combination of docetaxel and reovirus infection (16).

To confirm that docetaxel is acting by targeting MTs, we asked whether paclitaxel, another MT-stabilizing drug, also acts synergistically with metformin to increase cell death. For each cell line, the drug combination was more effective at decreasing cell number than either drug individually over a range of concentrations (Figure 2, measurements from 72 hr drug incubations). From these data the calculated CI values indicated synergy between paclitaxel and metformin in each cell line at the ED50 (Tables 1, 2). The drug combination shifted toward antagonism at higher concentrations, similar to the combination of docetaxel and metaformin.

Although we were able to measure synergy between MT stabilizing drugs and metformin, we were surprised by how relatively insensitive each cell line was to the MT-targeting drugs. Therefore, we next assayed cell cycle distributions to ask whether LNCaP and PC-3 cells were responding to docetaxel by blocking in mitosis. DNA content per cell was measured by flow cytometry. LNCaP cells incubated in 50 nM docetaxel (approximately one-half the ED50 for cell death) for 24 hrs showed a significant increase in the number of cells with 4N DNA content, but the cell cycle block was more pronounced in PC-3 cells, where nearly all cells had a 4N DNA content (Figure 3 A,B). For each cell line, incubation for 48 hours in docetaxel reduced the number of cells with 2N DNA content, and produced a large increase in the number cells with <2N DNA content, consistent with increased numbers of dying cells (Figure 3 A,B). Cells were also examined by fixation and staining for tubulin and DNA (Figure 4). In each cell line, docetaxel treatment resulted in formation of multipolar spindles and bundles of MTs, consistent with the greater MT stabilization. Therefore, both lines were responding to docetaxel with the expected mitotic block.

Metformin treatment did not appear to have a major impact on cell cycle progression, as observed by flow cytometry (Figure 3) and immunofluorescence (Figure 4). The metformin concentrations tested here, 1.5 - 3 mM, were lower than that shown previously to block cells in G1 (5 mM; 6).

Metformin also acts synergistically with a MT depolymerizing drug, nocodazole, to inhibit cell proliferation

To determine whether the observed synergy between MT stabilizing drugs and metformin resulted from MT stability (present throughout the cell cycle) or a cell cycle block during mitosis, we treated the two cell lines with nocodazole, which acts oppositely from docetaxel or paclitaxel and depolymerizes MTs, but also blocks cells in mitosis. Each cell line responded to 500 nM nocodazole with a block in G2/M, as measured by flow cytometry (Figure 5 A,B). Treatment of each cell line with a range of nocodazole and/or metformin concentrations inhibited cell proliferation (Figure 5 C,D) with a profile similar to that observed with docetaxel or paclitaxel with metformin. As observed with the MT stabilizing drugs, nocodazole was only moderately effective at killing cells in these lines, at least after 48 hr incubation. Metformin treatment resulted in greater cell death (or inhibition of cell proliferation) before reaching a plateau. The CI values calculated from these data indicate synergy between nocodazole and metformin at the ED75 in LNCaP cells and at the ED50 in PC-3 cells (Tables 1,2). MT stabilizers (docetaxel or paclitaxel) generally showed greater synergy with metformin than did MT depolymerization (nocodazole), as summarized in Tables 1,2.

Docetaxel synergizes with 2-DG to inhibit cell proliferation

To further test the hypothesis that MT-targeting drugs act in synergy with metabolic inhibition, we tested a second metabolic inhibitor, 2-DG, a competitive inhibitor of hexokinase, which catalyzes the first step of glycolysis. As shown in Figure 6, incubation in 2-DG reduced cell proliferation over a range of concentrations from 5 - 80 mM in both LNCaP and PC-3 lines. In combination with docetaxel, 2-DG-treated cells showed reduced cell proliferation, which was observed most prominently at the lower drug levels tested (Figure 6). The CI values at the ED50 for docetaxel and 2-DG indicated synergistic activity in both LNCaP and PC-3 cell lines (Tables 1,2). At higher doses these drugs shifted toward antagonistic activity. By flow cytometry we did not observe a cell cycle block in 2-DG (20 mM) treated cells (Figure 6), but treatment with both 2-DG and docetaxel resulted in a large fraction of cells remaining in G1, even in PC-3 cells, where docetaxel shifts almost all cells to G2/M phases within the 24 hour drug incubation (see Figure 3B). The flow cytometry data indicate that the combination of 2-DG and docetaxel may function synergistically by effecting more than one cell cycle stage. Others have reported cell cycle blocks in cells treated with 2-DG alone, but these blocks were

observed after longer incubations (4 days, 5 mM), and varied between a G1 block and G2/M block, depending on cell line (17).

Conclusion:

The synergies observed between MT-targeted drugs and two metabolic inhibitors indicate that these drug combinations could provide more effective tumor cell killing than either drug alone. In particular, docetaxel, currently the best chemotherapeutic option for mCRPC, was more effective at cell killing when combined with either metformin or 2-DG in two commonly used prostate cancer lines. A dual metabolic inhibition, combining treatment with both metformin and 2-DG, also resulted in greater cell death in the LNCaP prostate cancer cell line (6). Another treatment showing synergy in prostate cancer cells combined docetaxel and oncolytic reovirus infection (16).

The molecular mechanisms underlying drug synergies observed in prostate cancer cell lines, combining docetaxel with either metabolic inhibitors or reovirus infection (16), are not known. We attempted to test whether the synergies we observed were due to a mitotic block (produced by all of the MT-targeting drugs used here) or by an MT-dependent effect, independent of the cell cycle. LNCaP and PC-3 cells were treated with the cyclin-dependent kinase 1 inhibitor RO-3306 (18), which should block cell cycle progression just prior to mitotic entry. LNCaP were not blocked in mitosis by this drug, and in both cell lines we observed very little cell death after 48 h incubation in RO-3306, making it impossible to test for synergy with metabolic inhibitors. A mechanism independent of the cell cycle has been proposed from experiments in breast cancer cell lines and suggests that the combined treatment of paclitaxel and 2-DG induces cell death by causing oxidative stress (19). It is possible that a similar mechanism is responsible for the synergies observed in prostate cancer cells.

A clinical trial to test the combination of metformin and docetaxel in mCRPC is currently ongoing (20). While our cell-based studies suggest that combination therapy will be successful clinically, we note that metabolic inhibition may not always be effective in all cancers or in all patients, as discussed by Yamaguchi and Perkins (20).

Abbreviations:

2-DG - 2-deoxy-glucose
CI - combination index
ED - effective dose
FACs - Fluoresence-activated cell sorting
mCRPC - metastatic castrate-resistant prostate cancer
MT - microtubule
MTS - (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)

Competing Interests: The authors declare that they have no competing interests.

Authors' Contributions: BKC conducted experiments; BKC and LC designed experiments, analyzed data, and wrote the manuscript.

Acknowledgements: The authors are indebted to Lee Graham for his assistance with FACs analysis; Sara Lynn Farwell for providing insight into calculations of drug synergies; and Victoria Caruso-Silva and Dr. Robert Skibbens for providing advice during the course of these experiments.

Grant Support: Supported by a Hypothesis-Exploration Grant from the Prostate Cancer Research Program of the Congressionally Directed Medical Research Program (CDMRP) to LC.

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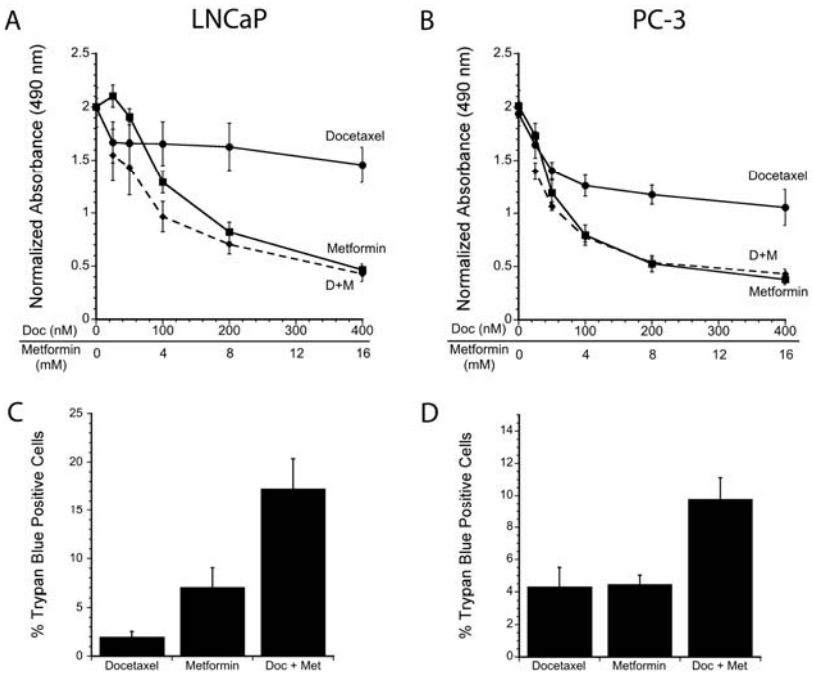

Figure 1: Metformin enhances the cell death caused by docetaxel treatment of LNCaP and PC-3 cell lines. (A, B) LNCaP (A) and PC-3 (B) cell lines were treated with docetaxel (0-400 nM), metformin (0-16 mM), or the two drugs in a constant ratio checkerboard design. For each experiment, each drug concentration or combination was tested in triplicate and cells were incubated for 48 hrs after addition of drugs. As a control, cells were incubated in 0.1% DMSO. Cell viability was measured by MTS assay and results were normalized to pool experiments. Plots show the combined data from 3 (LNCaP) or 4 (PC-3) independent experiments, additional experiments including different concentration ranges gave the same general patterns. (C, D) The percent cell death (Trypan blue positive cells) for LNCaP (C) and PC-3 (D) lines. Cells were incubated for 48 hrs in docetaxel (50 nM; approximately one-half the ED50 for cell death) and/or metaformin (1.5 mM; approximately one-half the ED50 for cell death). The percent trypan blue positive cells measured in DMSO-treated samples was subtracted from each experimental condition. Doc, D = docetaxel; Met, M = metformin

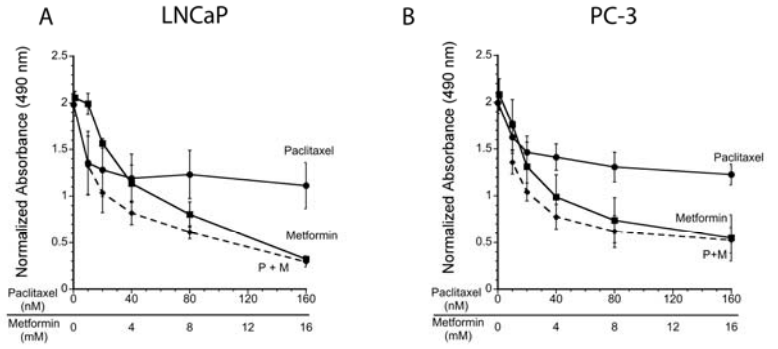

Figure 2: Metformin increases the cell death caused by paclitaxel in LNCaP and PC-3 cells. (A, B) LNCaP (A) and PC-3 (B) cell lines were treated with paclitaxel (0-160 nM), metformin (0-16 mM), or the two drugs in a constant ratio checkerboard design. Each drug concentration or combination was tested in triplicate in each experiment and cells were incubated for 72 hrs after addition of drugs. As a control, cells were incubated with 0.1% DMSO. Cell viability was measured by MTS assay and results from 3 independent experiments were normalized to pool experiments. P= paclitaxel; M = metformin

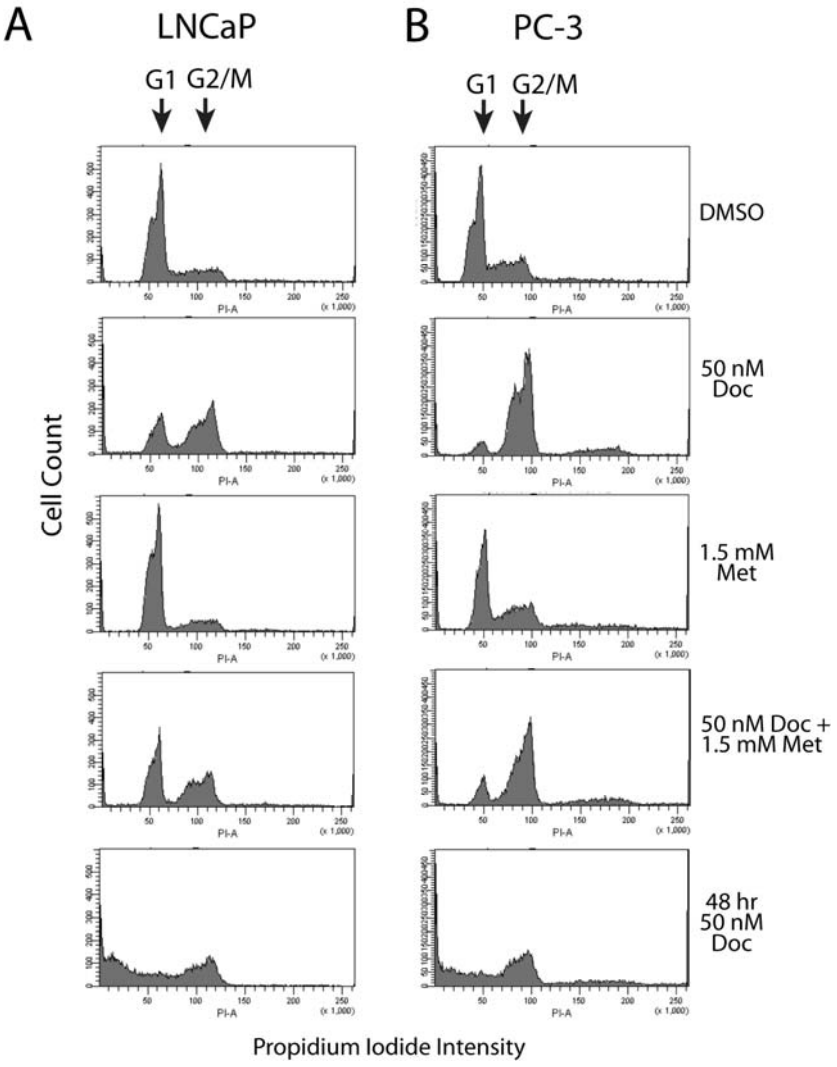

Figure 3: Cell cycle profiles of LNCaP and PC-3 cells treated with docetaxel and/or metformin. (A,B) DNA content of LNCaP (A) and PC-3 (B) cells measured by flow cytometry. Unless indicated otherwise, cells were incubated in drug containing medium for 24 hr. As a control, cells were incubated with 0.1% DMSO. Cells were fixed and DNA labeled with propidium iodide. Each experiment was repeated three times and plots shown are from representative experiments. Drug concentrations were approximately half of the estimated EC50 for each drug alone. Doc = docetaxel; Met = metformin.

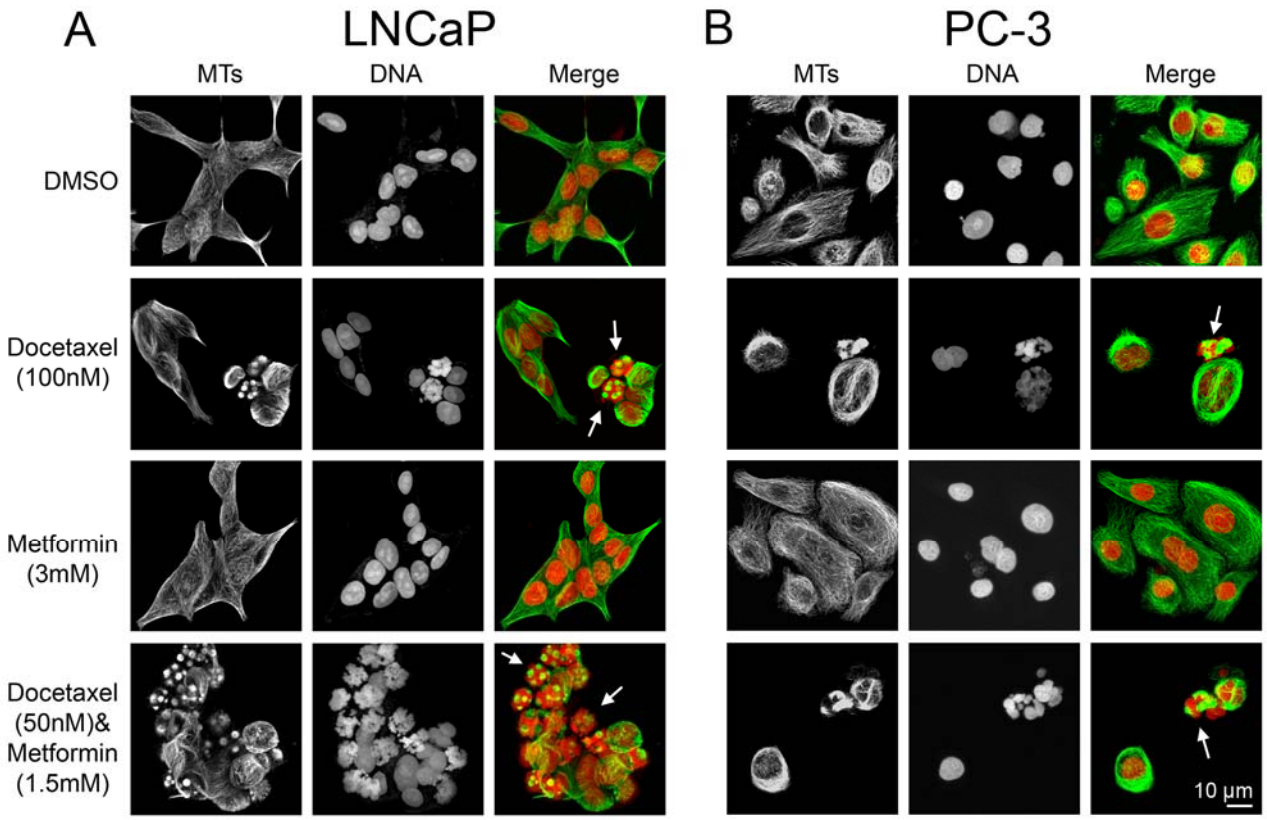

Figure 4: Docetaxel treatment resulted in multipolar spindle formation in LNCaP and PC-3 cells. Each cell line was incubated for 24 hr in DMSO, docetaxel or metformin and then fixed and stained with an antibody against α-tubulin (green in merged images) and propidium iodide (red in merged images). Representative images from each treatment are shown. Docetaxel, alone or in combination with metformin, resulted in a large increase in multipolar spindles (Arrows in merged images). Metformin-treated cells appeared to have MT organization indistinguishable from DMSO treated cells. Scale bar = 10 µm.

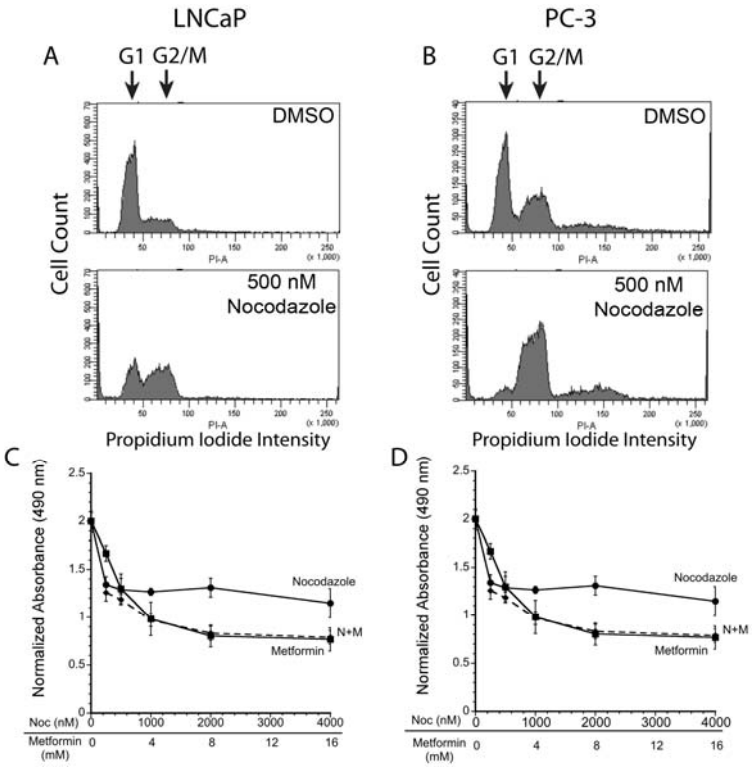

Figure 5: Metformin slightly enhanced nocodazole-induced cell death in LNCaP and PC-3 cell lines. (A,B) DNA content, measured by flow cytometry, for LNCaP (A) and PC-3 (B) cells after 24 hr incubation in 500 nM nocodazole, a MT-depolymerizing drug. For each cell line, the peak at 4N DNA content (G2/M phases) was increased significantly by drug treatment, indicating that each line was responding with the expected mitotic block. (C,D) Cell number decreased significantly in cells treated with nocodazole (0-4000nM) and/or metformin (0-16mM). Each drug concentration was assayed in triplicate 48 hrs after drug addition. As a control, cells were incubated in 0.1% DMSO. Plots show data pooled from 3 independent experiments. At the lower concentrations tested, nocodazole and metformin caused slightly more cell death than either drug separately. Noc, N = nocodazole; M = metformin.

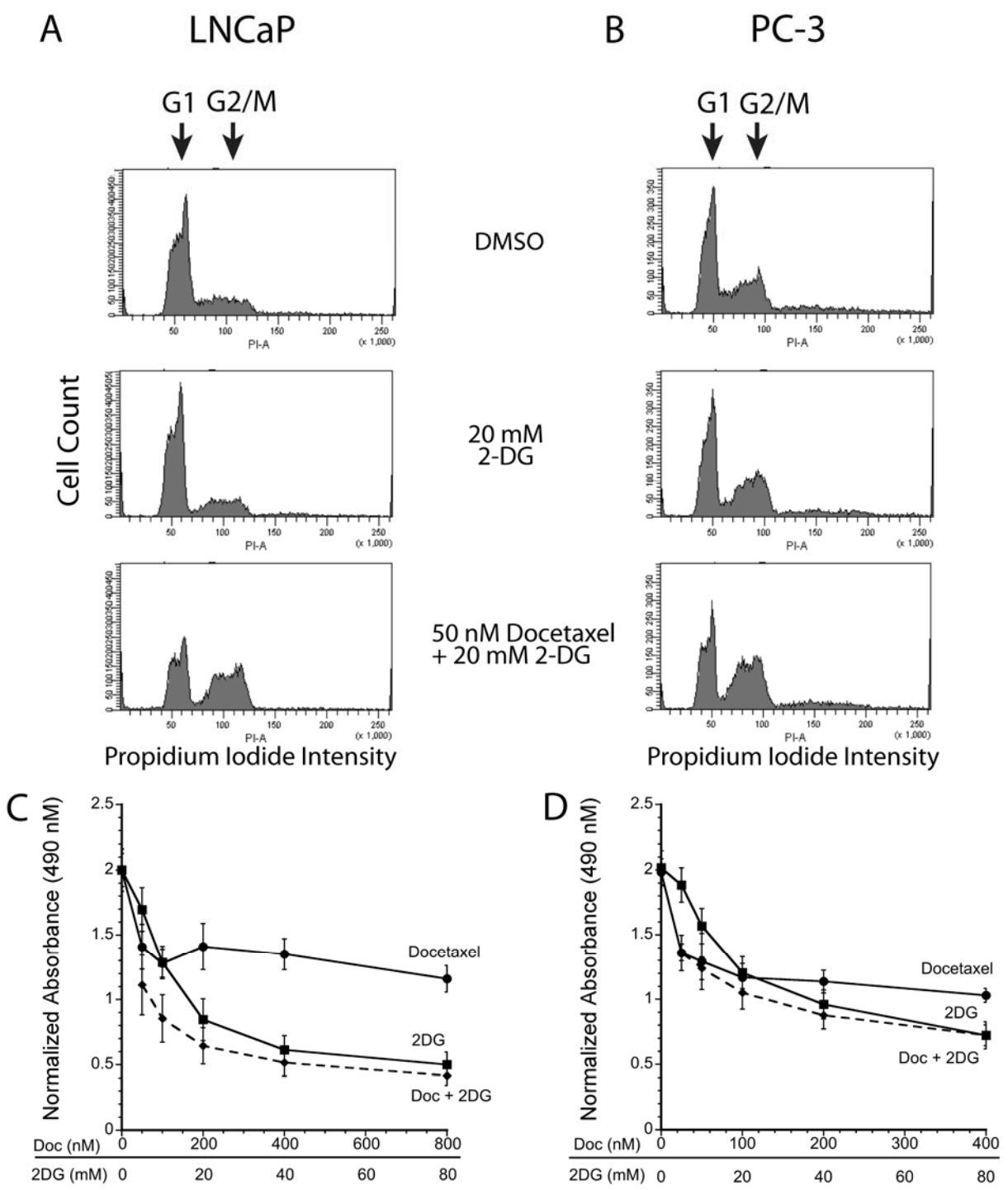

Figure 6: 2-DG enhances the cell death caused by docetaxel in LNCaP and PC-3 cells. (A,B) DNA content of LNCaP (A) and PC-3 cells incubated for 24 hrs in the indicated concentrations of 2-DG and/or docetaxel. No significant change in cell cycle distribution was noted for either cell line treated with 2-DG, while the mitotic block (increased numbers of cells with 4N DNA content, labeled G2/M) was observed after incubation in the combination of docetaxel and 2-DG. Drug concentrations are approximately half the ED50 value. (C,D) Cell number measured by MTS assay for LNCaP (C) and PC-3 (D) cells after 48 hr incubation in the indicated drugs. As a control, cells were incubated in 0.1% DMSO. Plots show normalized data averaged for 4 experiments in LNCaP cells and 3 experiments in PC-3 cells. Additional experiments covering a broader concentration range showed the same patterns. Doc = docetaxel.

Tables

Table 1: Combination Index values for LNCaP cells treated with MT-targeted drugs and metabolic inhibitors

Drug combination	ED50	ED75	ED90	r^2	n
Docetaxel, Metformin	0.86 ± 0.1	1.25 ± 0.16	1.98 ± 0.51	0.98	5
Paclitaxel, Metformin	0.63 ± 0.2	1.19 ± 0.33	3.00 ± 2.59	0.95	3
Nocodazole, Metformin	0.79 ± 0.19	3.31 ± 1.04	16.48 ± 12.65	0.92	3
Docetaxel, 2-DG	0.87 ± 0.18	2.08 ± 1.24	7.4 ± 7.29	0.99	5

Combination index values ± SD for the drug combinations listed.
Number of independent experiments, n.

Table 2: Combination Index values for PC-3 cells treated with MT-targeted drugs and metabolic inhibitors.

Drug combination	ED50	ED75	ED90	r^2	n
Docetaxel, Metformin	0.67 ± 0.24	0.90 ± 0.15	1.49 ± 0.72	0.96	7
Paclitaxel, Metformin	0.72 ± 0.29	0.78 ± 0.22	1.66 ± 0.53	0.96	3
Nocodazole, Metformin	0.99 ± 0.89	0.63 ± 0.1	3.93 ± 3.29	0.93	3
Docetaxel, 2-DG	0.37 ± 0.14	0.72 ± 0.12	1.68 ± 0.79	0.97	6

Combination index values ± SD for the drug combinations listed.
Number of independent experiments, n.